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Characterization of the Lipopolysaccharide O Antigens of *Actinobacillus pleuropneumoniae* Serotypes 9 and 11: Antigenic Relationships among Serotypes 9, 11, and 1†

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The antigenic lipopolysaccharide O polysaccharides of capsular serotypes 9 and 11 were examined by chemical, immunological, and nuclear magnetic resonance methods. Immunodiffusion tests carried out on these O antigens indicated that both contained common epitopes which were also shared by *Actinobacillus pleuropneumoniae* serotype 1. Chemical analysis and high-field nuclear magnetic resonance spectroscopy showed that the O antigens of serotypes 9 and 11 were high-molecular-weight polymers consisting of a backbone of repeating trisaccharide units composed of α-L-rhamnopyranosyl and α-D-glucopyranosyl residues (2:1). One of the α-L-rhamnose units forms a branch point and is stoichiometrically substituted with terminal 2-acetamido-2-deoxy-β-D-glucose residues in the serotype 11 O polysaccharide, but only to the extent of 25% in the serotype 9 O polysaccharide. Thus, the serotype 9 O polysaccharide contains two different repeating units: a tetrasaccharide unit with the same structure as that of the serotype 11 O polysaccharide and a trisaccharide unit:

$$\begin{array}{l}\rightarrow 6)\text{-}\alpha\text{-D-Glc}_p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}_p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}_p\text{-}(1\rightarrow \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad 3 \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \uparrow \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \mathrm{R}\end{array}$$

where R = β-D-Glc$_p$NAc for serotype 1 and 11 O polysaccharides, and R = H (75%) and R = β-D-Glc$_p$NAc (25%) for serotype 9. The structure of the previously determined serotype 1 O polysaccharide (E. Altman, J.-R. Brisson, and M. B. Perry, Biochem. Cell. Biol. 64:17–25, 1986) is identical to that of the serotype 11 O polysaccharide. We propose a more complete serotyping scheme for *A. pleuropneumoniae* which includes designation of both the capsular (K) and O antigens.

Actinobacillus pleuropneumoniae is a gram-negative, pleomorphic rod which causes a highly contagious disease in pigs. The occurrence of *A. pleuropneumoniae*-associated pleuropneumonia has increased since 1976 (29) because of intensified pig production, and *A. pleuropneumoniae* lipopolysaccharide (LPS) is recognized as an important virulence factor (30). In attempts to combat the disease, serological methods of identification of infected animals based on the identification and characterization of serotype-specific antigens would greatly assist in solving the problem. Information on the structures of the LPS O antigens of *A. pleuropneumoniae* involved as virulence factors, in protection, and in serodiagnosis could provide data leading to the rational development of suitable, protective vaccines and the production of specific serotyping and serodiagnostic reagents.

Currently, 12 serotypes of *A. pleuropneumoniae* based on capsular polysaccharides (CPS) are recognized (26). Capsular serotypes 1, 5, and 7 are the predominant disease isolates from *A. pleuropneumoniae* outbreaks in the United States and Canada (28), whereas serotype 9 is predominant in The Netherlands (19). Serological cross-reactivity between *A. pleuropneumoniae* serotypes 1 and 9 has been detected by complement fixation and gel diffusion methods (25). This cross-reactivity was attributed to common polysaccharide antigens by Mittal (24), who also concluded that these serotypes do not share CPS epitopes but that the cross-reactivity observed may be due to somatic antigens. Analyses of the CPS of serotypes 1 and 9 carried out in our laboratory demonstrated that their structures, while having some similarities, contained unique epitopes (27).

We report here that the LPS O antigens of serotypes 1, 9, and the recently identified serotype 11 (26) express common carbohydrate epitopes. In addition, the serotype 9 O antigen contains unique epitopes. In order to identify the molecular features associated with these epitopes, structural determination of the O-polysaccharide antigens (O-PS) of serotypes 9 and 11 was undertaken. The structure of the O-PS from the serotype 1 LPS was previously reported by Altman et al. (1) to consist of branched tetrasaccharide repeating units with the structure

$$\begin{array}{l}\rightarrow 6)\text{-}\alpha\text{-D-Glc}_p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}_p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}_p\text{-}(1\rightarrow \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad 3 \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \uparrow \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad 1 \\ \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad \beta\text{-D-Glc}_p\text{NAc}\end{array}$$

The structure of the LPS O chain of the capsular serotype 11 is identical to that of serotype 1, whereas that of the serotype 9, although similar, differs in that the common

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trisaccharide backbone is only partially substituted by terminal 2-acetamido-2-deoxy-β-D-glucose residues. Our studies indicate that a more detailed serotyping scheme which would include O-antigenic factors would be warranted (27).

MATERIALS AND METHODS

Organism and growth conditions. *A. pleuropneumoniae* serotypes 9 (strain CVJ 13261; NRCC 4264) and 11 (strain 456153; NRCC 4330) were obtained from S. Larivière, University of Montreal, Saint Hyacinthe, Quebec, Canada. The cells were grown in Bacto PPLO broth supplemented with 5% horse serum (Bockneck), glucose (to a final concentration of 1%), and NAD (Sigma Chemical) (to a final concentration of 50 μg/ml), with a 28-liter fermentor (New Brunswick Scientific) at 36 ± 1°C overnight. The cells were then killed with 0.75% phenol (final concentration), prior to being harvested with a Sharples continuous centrifuge.

LPS extraction and purification of O-PS. Cells of *A. pleuropneumoniae* serotypes 9 (280 g [wet weight]) and 11 (400 g [wet weight]) were washed (2% saline), digested with lysozyme, RNase, and DNase, and then extracted with hot aqueous phenol (17). The aqueous and phenol layers were dialyzed against running tap water until phenol free and then lyophilized. The lyophilizates were taken up in 0.9% (wt/vol) saline and, following removal of insoluble material by low-speed centrifugation, the clear solutions were ultracentrifuged (105,000 × *g*, 4°C, 12 h) to give precipitated LPS gels, which were dissolved in distilled water and lyophilized. The LPSs from serotypes 9 and 11 were obtained in yields of 2.4 and 3.0 g, respectively.

For preparation of the O-PS, solutions of LPS (300 mg each) in 100 ml of 1% acetic acid were heated to 100°C for 3 h and the precipitated lipid A (~37% from serotype 9 and ~21% from serotype 11) was removed by low-speed centrifugation. The supernatants were lyophilized and then taken up in 0.05 M pyridinium acetate (pH 4.7; 10 ml) and fractionated by gel filtration on a Sephadex G-50 column (Pharmacia; 2 by 90 cm) with the same buffer as eluant. Fractions of the eluate (10 ml each) were collected and analyzed colorimetrically for neutral aldose (13), aminoglycose (15), phosphate (11), and 3-deoxy-D-*manno*-2-octulosonic acid (3). The gel filtration properties of the eluted materials are expressed in terms of their distribution coefficients K_{av}. $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the elution of volume of the specific material, V_o is the void volume, and V_t is the total volume of the system.

The O-PS were further purified by DEAE-Sephacel chromatography. After being dissolved in water, the samples (20 to 30 mg) were applied to a column (1.2 by 35 cm) which was first irrigated with 0.05 M Tris-HCl buffer (pH 7.2; 40 ml) to obtain the O-PS and then with a 0-0.5 M sodium chloride gradient in the same buffer. Fractions (1 ml each) were collected.

Production and absorption of antisera. Rabbit antisera to *A. pleuropneumoniae* serotypes 1 and 9 were prepared by injecting formalized suspensions of whole cells (approximately 2×10^8 CFU/ml) in 0.01 M phosphate-buffered saline (PBS) buffer (pH 7.0) with Thimersol (1:10,000) into New Zealand White rabbits, weighing between 2.2 and 2.5 kg. Following preimmunization, 0.25 ml of a 1:4 dilution of the suspensions was introduced subcutaneously, and then the same volume of the 1:4 dilution was injected 3 to 4 days later by the intramuscular route. Injections 3 and 4 were 1:2 dilutions, and the volumes were 0.5 and 1.0 ml, respectively. The last two sets of inoculations were of undiluted suspensions, in volumes of 1.5 ml and 2.0 ml, respectively. All four of these last injections were given intravenously. The rabbits were test bled 3 to 4 days after the final injection. Final bleeding was done after gel diffusion plates were run to confirm the efficacy of the antisera.

Serotype 11 LPS was insolubilized according to the method of Eskenazy et al. (14). A suspension of the insolubilized LPS (100 mg/ml) was added to a solution of purified (18) serotype 9 antiserum (27 mg) in PBS (10 ml), and the mixture was stirred for 1 h at room temperature. The precipitate was removed by low-speed centrifugation, a further 0.5 ml of insolubilized LPS suspension was added to the supernatant, and the mixture was stirred for 30 min. After removal of the final precipitate, the supernatant (5.0 ml) was concentrated (×50 with an Amicon concentrator).

ID tests. The immunodiffusion (ID) tests were carried out on disposable plastic plates with 2% agar (Noble agar; Difco Laboratories) dissolved in water containing 1.0% NaCl and 7.6% glycine. The reagent wells (2 mm deep; 4-mm diameter) were placed at a distance of 6 mm from each other and the central well. The plates were incubated at 4°C in a humid atmosphere and were examined daily for 2 days.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). LPS samples (1 μg each) were analyzed in 14% polyacrylamide gels by electrophoresis in the presence of 2% SDS. Bands were detected by the silver staining method of Tsai and Frasch (32).

Analytical methods. The quantitative colorimetric methods used were (i) phenol-sulfuric acid for neutral glycoses (13), (ii) the modified Elson-Morgan method for aminoglycoses (15), (iii) the method of Chen et al. for phosphate (11), and (iv) the periodate oxidation-thiobarbituric acid method for 3-deoxy-D-*manno*-2-octulosonic acid (3).

For analysis of constituent sugars, samples (1 mg each) of polysaccharide were hydrolyzed with 4 M trifluoroacetic acid for 1 h at 125°C. The excess acid was removed by evaporation under a stream of nitrogen, and the identities of the glycoses were determined by gas-liquid chromatography-mass spectrometry (GLC-MS) of their derived alditol acetates (16).

The configurations of the glycoses were established by GLC-MS of their acetylated (*R*)-2-octyl glycosides (23).

Analytical GLC-MS was performed with a Hewlett-Packard model 5958B gas chromatograph, fitted with an OV-17 fused silica capillary column (Quadrex Corp.), in the electron impact mode, with an ionization potential of 70 eV. The following temperature programs were employed: (i) for alditol acetates, 180°C for 2 min and then 4°C/min to 240°C; (ii) for partially methylated alditol acetates, 180°C for 2 min and then 2°C/min to 240°C; and (iii) for acetylated (*R*)-2-octyl glycoside derivatives, 180°C for 2 min and then 6°C/min to 240°C.

Methylation analysis. Methylation of samples (2 mg each) was carried out by the method of Ciucanu and Kerek (12), and the products were isolated by partition between dichloromethane and water. The methylated products were hydrolyzed (4 M trifluoroacetic acid for 1 h at 125°C) and, following reduction ($NaBD_4$), were analyzed by GLC-MS as the acetylated alditol derivatives.

NMR spectroscopy. All measurements were made on solutions in D_2O with a Bruker AM-500 or AMX-500 spectrometer.

Proton spectra recorded at 500 MHz were obtained by using a spectral width of 22 kHz, a block with 16,000 datum points, and a 90° pulse. Chemical shifts are expressed relative to internal acetone (δ_H, 2.225 ppm).

Broad-band, proton-decoupled ^{13}C spectra were obtained at 125 MHz with a spectral width of 31 kHz, a block with 32,000 datum points, and a 90° pulse employing WALTZ decoupling (31). The internal reference was acetone (δ_C, 31.07 ppm).

Two-dimensional homonuclear chemical shift-correlated spectroscopy and nuclear Overhauser enhancement spectroscopy were carried out as previously described (6) over a sweep width of 2,212 Hz. Heteronuclear ^{13}C-^1H chemical-shift correlations were measured in the ^1H-detected mode via multiple-quantum coherence ($^1H\{^{13}C\}$HMQC) (5) with a Bruker 5-mm inverse broad-band probe with reverse electronics as previously described (7).

RESULTS

Isolation and purification of the O-PS of *A. pleuropneumoniae* serotypes 9 and 11. The LPSs from cells of *A. pleuropneumoniae* serotypes 9 and 11 were obtained by a modified lysozyme phenol-water procedure (17), followed by ultracentrifugation. The serotype 9 LPS was found solely in the aqueous layer (0.9%, based on wet weight of cells), whereas the serotype 11 LPS was distributed between the aqueous (0.4%) and phenol (0.4%) layers. ^1H-nuclear magnetic resonance (NMR) analysis showed that the O-PS of the LPS from the aqueous and phenol fractions of serotype 11 were identical. The spectra obtained for the LPS O-PS from aqueous fractions are shown in Fig. 1.

SDS-PAGE analyses of the LPS preparations showed similar ladderlike banding patterns that were indicative (32) of S-type LPS (data not shown).

Partial hydrolysis of each LPS gave insoluble lipid A (37% from serotype 9 and 21% from serotype 11). Gel filtration chromatography, on Sephadex G-50, of the respective water-soluble products afforded O-PS (K_{av}, 0.03 [21% from serotype 9 and 41% from serotype 11]), core oligosaccharide (K_{av}, 0.65 [18% from serotype 9 and 11% from serotype 11]), and a low-molecular-weight fraction (K_{av}, 0.97) which contained phosphate and 3-deoxy-D-*manno*-2-octulosonic acid.

The O-PS of serotypes 9 and 11 were further purified, to remove contaminating traces of CPS, by DEAE-Sephacel chromatography, in which they eluted with the neutral buffer (pH 7.2). The purified O antigens were free of protein impurities (Bio-Rad Protein Assay).

ID tests. Figure 2a shows the precipitin pattern observed in the ID assay with anti-serotype 1 serum, the O antigens of serotypes 1, 9, and 11, and the deamination product of the serotype 1 O antigen which lacks the terminal 2-acetamido-2-deoxy-β-D-glucose (β-D-Glc$_p$NAc) residue (1). The three native O antigens all showed identical precipitating properties, i.e., serotypes 1 and 9 (Fig. 2a [wells 4 and 3]) and serotypes 9 and 11 (Fig. 2a [wells 3 and 2]) gave continuous precipitin lines. On the other hand, the deaminated serotype 1 O antigen did not give a line of precipitation (Fig. 2a [well 1]).

Anti-serotype 9 serum produced precipitin lines against all of the O antigens, including the deaminated serotype 1, after 24 h at 4°C (Fig. 2b). After 48 h, the deaminated serotype 1 and serotype 9 O antigens (Fig. 2c [wells 1 and 3]) gave more-diffuse precipitin lines of "partial identity" with those seen with the serotype 1 and 11 O antigens (Fig. 2c [wells 4 and 2]).

Absorption of the anti-serotype 9 serum with insolubilized (14) serotype 11 LPS resulted in the disappearance of a precipitin line with serotype 1 and 11 O antigens (Fig. 2d [wells 4 and 2]). A continuous precipitin line of identity was still observed, however, with the O antigen of serotype 9 and the deaminated product of the serotype 1 O antigen (Fig. 2d [wells 3 and 1]).

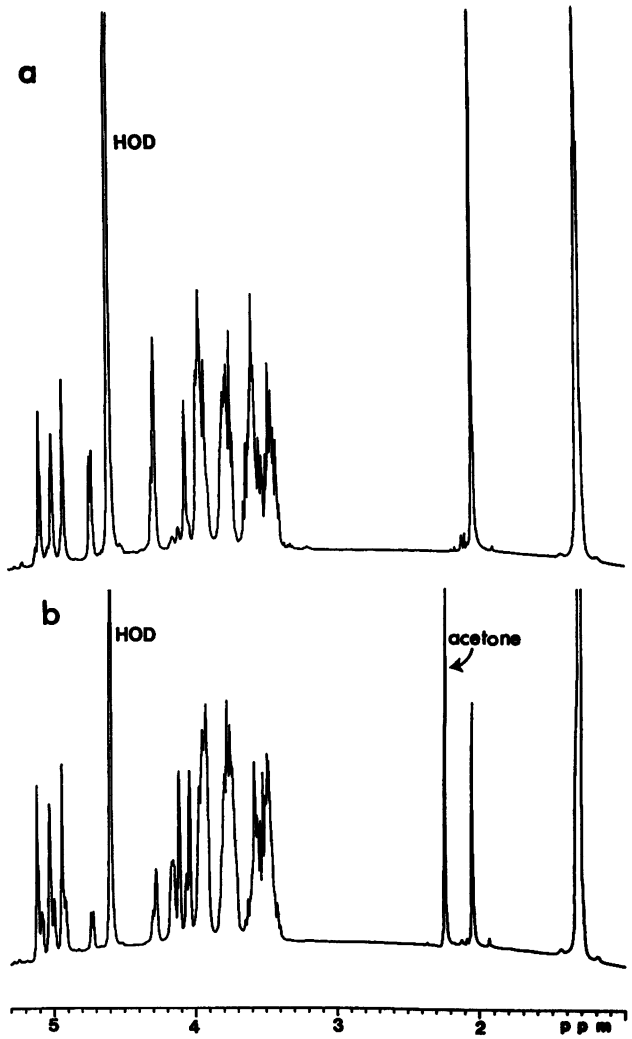


FIG. 1. Proton-NMR spectra of the *A. pleuropneumoniae* O-PS recorded at 42°C. (a) Serotype 11; (b) serotype 9. HOD, ^1H resonance from deuterated water.

Characterization of the O-PS. The O-PS of serotypes 9 and 11 had $[\alpha]_D$ of +38° (c, 1.0 [water]) and +17° (c, 1.0 [water]), respectively. Analysis found for serotype 9 O-PS was as follows: C, 39.3; H, 6.4; N, 0.7; ash, 0.9%; for serotype 11 O-PS, analysis found was as follows: C, 38.6; H, 6.4; N, 1.6; ash, 2.4%. Total acid hydrolysis (4 M trifluoroacetic acid for 1 h at 125°C) indicated that each O chain contained L-rhamnose, D-glucose, and D-glucosamine. However, relative to that of glucose, the amount of glucosamine present in the serotype 9 O-PS was only one-third of that present in the serotype 11 O-PS.

The absolute configurations of the glycoses were determined by the method of Leontein et al. (23).

Methylation of the serotype 9 and 11 O-PS followed by hydrolysis and analysis of the resulting partially methylated glycoses as their alditol acetates (16) gave the results shown in Table 1.

The ^1H-NMR spectra of the serotype 9 and 11 O-PS showed a number of similar features (Fig. 1). The high-field

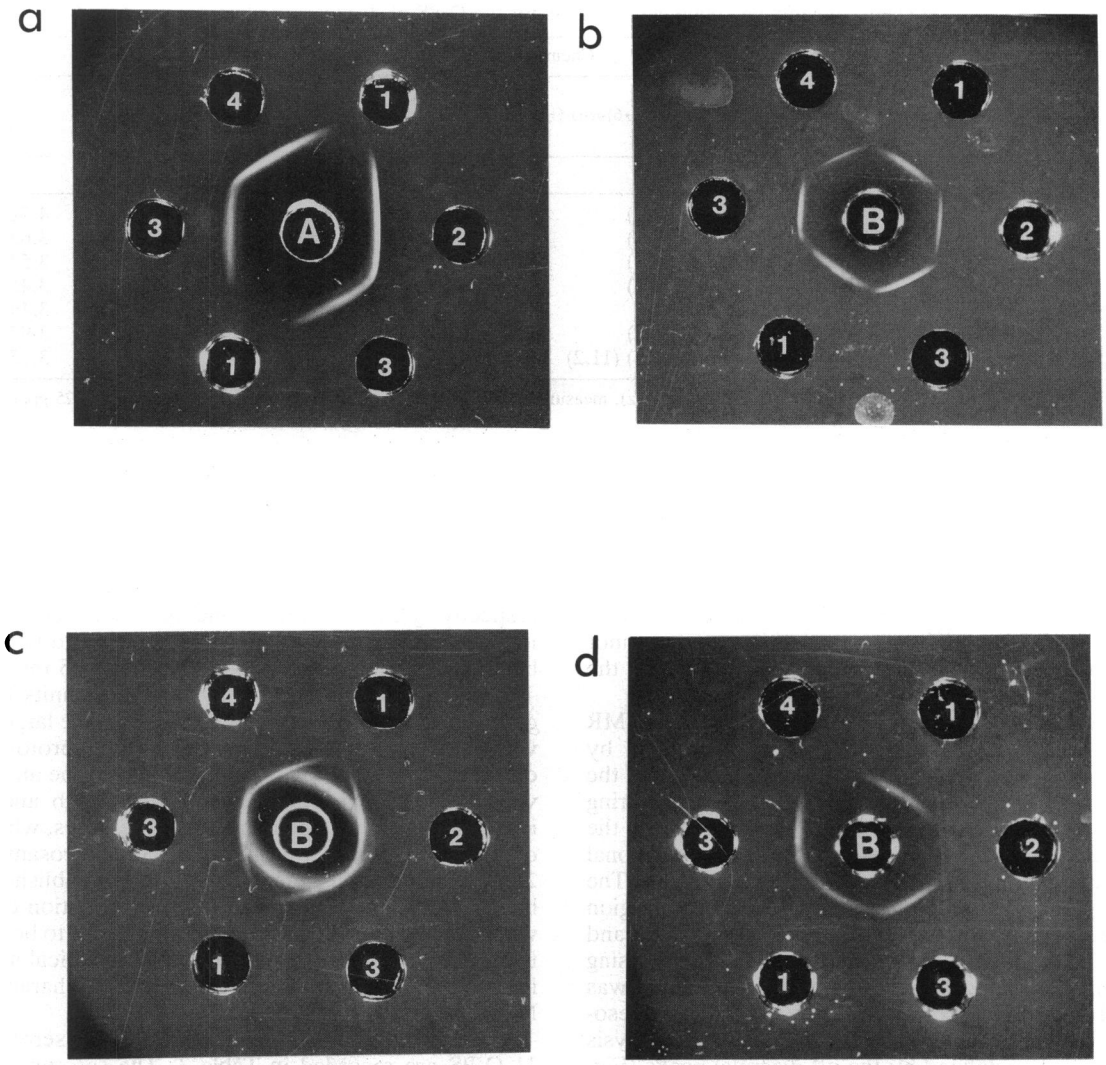


FIG. 2. Immunodiffusion plates. *A. pleuropneumoniae* serotype 1 antiserum (10 μl) (well A) (a), serotype 9 antiserum (10 μl) after 24 h (b [well B]) and after 48 h (c) and serotype 11 LPS absorbed serotype 9 antiserum after 48 h (d). Wells: 1, deaminated serotype 1 O-PS; 2, serotype 11 O-PS; 3, serotype 9 O-PS; 4, serotype 1 O-PS. Wells 1 to 4 each contained 25 μg of O-PS in 10 μl of distilled water.

region of each spectrum showed doublets (J, ~7 Hz) at 1.33 and 1.31 ppm for the methyl groups of two deoxy sugars and a singlet at 2.03 ppm for the methyl protons of an *N*-acetyl substituent. Although qualitatively similar, the relative intensities of the latter signal were however quite different.

TABLE 1. Methylation analysis data from *A. pleuropneumoniae* serotype 9 and 11 O-PS

Methylated sugar[a] (as alditol acetates)	T_M[b]	Molar ratio for serotype	
		9	11
3,4-Rha	1.0	1.5	1.0
4-Rha	1.4	0.2	0.9
2,3,4-Glc	1.7	1.0	1.0
2,3,4,6-GlcNAc	2.8	0.3	0.8

[a] 2,3,4-Glc is 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol, etc.
[b] Retention times (T_M) are quoted relative to that of 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylrhamnitol.

The serotype 11 O-PS showed four signals of equal signal areas for anomeric protons in the low-field region (5.11 to 4.72 ppm) of the spectrum (Fig. 1a). In contrast, the serotype 9 O-PS showed two groups of resonances in this region: a series of four minor peaks which have chemical shifts identical to those of the serotype 11 O-PS (Fig. 1b; Table 2), as well as a major group (relative signal areas, 4:1) consisting of three peaks.

The ^{13}C-NMR spectrum of the serotype 11 O-PS contained 25 resonances, one signal having an intensity of two (Table 3). This spectrum was indistinguishable from that produced by the *A. pleuropneumoniae* serotype 1 O-PS (1). The ^{13}C-NMR spectrum of the serotype 9 O-PS contained 18 major signals, together with 21 minor resonances which were less well resolved (Table 3). There were three major signals in the anomeric region at 100.5, 99.8, and 98.9 ppm and three minor resonances at 103.9, 100.4, and 98.5 ppm. Minor

TABLE 2. ^{1}H chemical shifts and coupling constants for the O-PS of *A. pleuropneumoniae* serotype 9[a]

Proton	Chemical shift for sugar residue: →2)-α-L-Rha$_p$-(1→ 3 ↑		→6)-α-D-Glc$_p$-(1→		→2)α-L-Rha$_f$-(1→		β-D-Glc$_p$ NAc-(1→
	a	a*	b	b*	c	c*	d*
H-1 ($J_{1,2}$)	5.11 (<3)	5.07 (<3)	5.03 (2.3)	4.99 (3.5)	4.94 (<3)	4.92 (<3)	4.72 (7.5)
H-2 ($J_{2,3}$)	4.11 (3.7)	4.27 (3.7)	3.56 (9.4)	3.52 (10.0)	4.03 (3.7)	4.06 (3.7)	3.63 (9.4)
H-3 ($J_{3,4}$)	3.91 (9.7)	3.95 (9.7)	3.76 (9.3)	3.74 (9.0)	3.92 (9.7)	3.93 (9.7)	3.57 (8.9)
H-4 ($J_{4,5}$)	3.52 (9.7)	3.58	3.47 (9.7)	3.56 (10.0)	3.47 (10.0)	~3.46	3.41 (9.4)
H-5	3.80	3.79	4.16	4.28	3.75	—[b]	3.46
H-6 ($J_{5,6}$)	1.32 (6.8)	~1.32	3.95 (~1)	—[b]	1.32 (6.8)	~1.32	3.97 (~1)
H-6′ ($J_{5,6}$) ($J_{6,6'}$)			3.71 (7.5) (11.2)	—[b]			3.75 (7.1) (10.5)

[a] Observed first-order chemical shifts and coupling constants (±1 Hz), measured at 42°C, using acetone as an internal reference (δ, 2.225 ppm).
[b] Unresolved.

resonances were also observed at 175.3 and 23.1 ppm for the C=O and CH_3 functionalities of the *N*-acetyl substituent and at 56.8 ppm for C-2 of a 2-acetamido-2-deoxyhexopyranose residue. Resonances at 17.81 and 17.76 (minor) and at 17.51 and 17.45 (major) were indicative of the presence of 6-deoxyhexose residues. The chemical shifts of the minor resonances were in close agreement with those of the serotype 11 O-PS.

Structure of the serotype 9 O-PS. The ^{1}H- and ^{13}C-NMR spectra of the serotype 9 O-PS were fully assigned by two-dimensional NMR methods (4, 5). A contour plot of the homonuclear chemical shift-correlated spectrum of the ring proton region is shown in Fig. 3b. In this spectrum, the diagonal peaks represent the conventional one-dimensional ^{1}H-NMR spectrum, and a projection is shown in Fig. 3a. The three paired groups of resonances in the anomeric region were labelled H-1a and H-1a* (major and minor), H-1b and H-1b*, and H-1c and H1-c* according to their decreasing chemical-shift values, and the minor peak at 4.72 ppm was labelled H-1d*. By being mapped from the anomeric resonances, the sugar residues were identified from an analysis of the connectivities defined by the off-diagonal peaks (Fig. 3b). The chemical-shift values and coupling constants are recorded in Table 2. The rhamnose (Rha) residues were readily identified from the observed small magnitude (≤4 Hz) of the vicinal couplings $J_{1,2}$ and $J_{2,3}$, which are indicative of a pyranose ring system having the *manno* configuration (2). Residues a, a*, c, and c* could be attributed to the Rha$_p$ units (Table 2). Furthermore, the high-field methyl doublet (relative area, ~6 H) at 1.32 ppm could be mapped into the connectivity pathways of residues a and c via cross-peaks to the H-5 resonances (3.80 and 3.75 ppm, respectively). However, the methyl resonances for the residues a* and c* were not resolved and could not be unambiguously correlated with the respective H-5 resonances.

Sugars which are present as pyranosyl units having the *gluco* configuration can be identified from the large observed values (8 to 10 Hz) of the vicinal ring proton coupling constants (2). After the chemical shift and the anomeric $J_{1,2}$ values were taken into account, residues b and b* were identified as α-linked glucopyranosyl residues, while residue d* was identified as a β-linked *N*-acetylglucosamine (Table 2). The latter assignment was firmly established from a heteronuclear ^{13}C-^{1}H chemical-shift correlation experiment which showed H-2 of this residue (3.63 ppm) to be correlated to the ^{13}C resonance at 56.8 ppm. ^{13}C chemical-shift values in the region of ca. 50 to 58 ppm are characteristic of N-substituted secondary carbons (10).

The ^{13}C chemical-shift assignments of the serotypes 9 and 11 O-PS are recorded in Table 3. The serotype 11 assignments were made by comparison with the published data for the serotype 1 O-PS (1), while ^{13}C chemical-shift correlation with the assigned proton resonances provided most of the ^{13}C resonance assignments for the serotype 9 O-PS.

The resonances for C-2 from the α-L-Rha$_p$ residues were significantly deshielded (77 to 79 ppm), indicating that each

TABLE 3. ^{13}C chemical shifts for the O-PS of *A. pleuropneumoniae* serotypes 9[a] and 11[b]

Carbon	Chemical shift for sugar residue: →2-α-L-Rha$_p$-(1→ 3 ↑		→6-α-D-Glc$_p$-(1→		→2-α-L-Rha$_p$-(1→		β-D-Glc$_p$NAc-(1→
	a	a*	b	b*	c	c*	d*
C-1	100.5	100.4	98.9	98.5	99.8	—[c]	103.9
C-2	77.8	76.7	72.2	72.3	79.5	78.9	56.8
C-3	70.5	78.9	73.6	73.8	70.8	70.9	74.9
C-4	73.1	70.1	70.4	72.4	73.0	—[c]	71.4
C-5	70.3	70.6	71.8	71.2	69.7	—[c]	76.6
C-6	17.5	17.8	67.3	—[c]	17.5	17.8	61.9

[a] Measured at 42°C with acetone as an internal reference (δ, 31.07 ppm).
[b] Chemical shifts for the sugar residues of serotype 11 O-PS are the same as those for the sugar residues of the tetrasaccharide repeating units of serotype 9 (± 0.1 ppm).
[c] Not resolved.

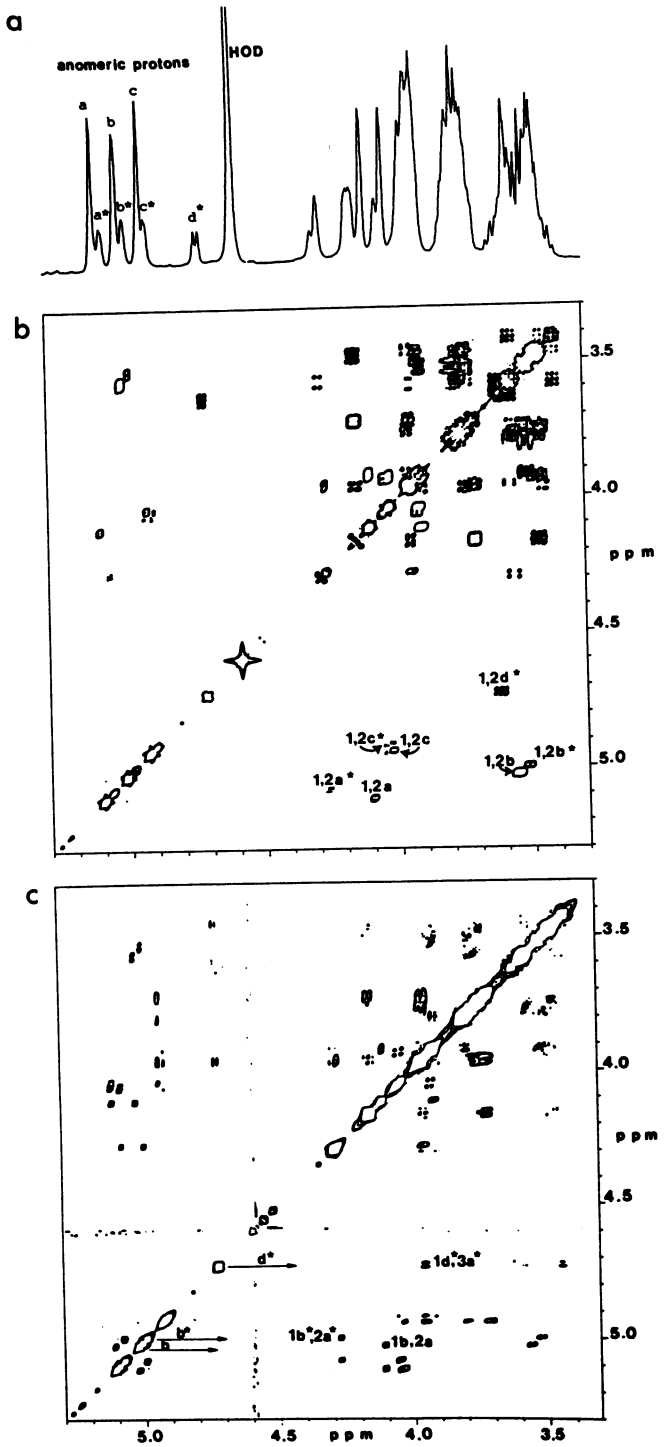


FIG. 3. (a) The one-dimensional ^{1}H-NMR spectrum of the ring proton region (5.30 to 3.30 ppm) of the *A. pleuropneumoniae* serotype 9 O-PS, recorded at 42°C. The anomeric protons are labelled as indicated in the text. (b) Contour plot of the chemical shift-correlated spectrum of the ring proton region. Cross-peaks relating the anomeric and H-2 resonances of each series are indicated. (c) Contour plot of the nuclear Overhauser enhancement spectrum of the ring proton region. Key interresidue NOEs for the two repeating units are indicated. HOD, ^{1}H resonance from deuterated water.

TABLE 4. NOE data from *A. pleuropneumoniae* serotype 9 O antigen

Observed proton	Intraresidue	Interresidue	Partial sequence
H-1a	H-2a	H-2c	a→c
H-1b	H-2b	H-2a	b→a
H-1c	H-2c	H-6b/6'b	c→b
H-1a*	H-2a*	H-2c*	a*→c*
H-1b*	H-2b*	H-2a*	b*→a*
H-1c*	H-2c*	H-6b*/6'b*[a]	c*→b*
H-1d*	H-3d*/H-5d*	H-3a*	d*→a*

[a] Resonances for H-6b/H-6* and H-6'b/H-6'* were not resolved.

of these residues was substituted at the corresponding position (8). Furthermore, the considerable downfield shift of C-3 of the residue a* (78.9 ppm) compared with that of residue a (70.5 ppm) indicates that the former α-L-Rha$_p$ residue is also substituted at the O-3 position.

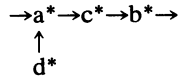
The methylene carbon resonance at 67.3 ppm (identified by a DEPT experiment) mapped into the ^{1}H spin system of the α-D-Glc$_p$ residue (b) via connectivities to the H-6 proton resonances (3.95 and 3.71 ppm). The downfield position of the C-6 resonance is indicative of substitution at the corresponding position, demonstrating the presence of →6)-α-D-Glc$_p$-(1→ units.

Comparison of the ^{13}C chemical shifts of the β-D-Glc$_p$NAc residue (d*) with published data (8) suggests that this sugar is present as a nonreducing terminal residue in the serotype 9 O-PS. Indeed, the chemical-shift data (Table 3) for the minor series of resonances correspond closely to those of the serotype 11 O-PS, suggesting that the branched tetrasaccharide unit present in the serotype 11 polysaccharide also forms a component of the serotype 9 O-PS.

The sequences of the sugar residues giving rise to the two groups of resonances (major and minor) in the serotype 9 O-PS were unequivocally established from nuclear Overhauser effect (NOE) measurements (21). To obtain this information, a two-dimensional experiment was carried out, and the contour plot of the ring proton region is shown in Fig. 3c. In this experiment, off-diagonal peaks indicate the existence of through-space connectivities between protons having an internuclear separation of ca. <3.0 Å (0.3 nm).

The occurrence of transglycosidic NOE demonstrated connectivities between sugar residues within the series of major resonances (Table 4), which established the linear partial sequence →a→c→b→, i.e., →2)-α-L-Rha$_p$-(1→2)-α-L-Rha$_p$-(1→6)-α-D-Glc$_p$-(1→. The same pattern could be demonstrated within the minor series (Table 4), establishing the sequence →a*→c*→b*→.

The only transglycosidic NOE observed for the anomeric proton of the β-D-Glc$_p$NAc residue (d*) was to H-3 of the α-L-Rha$_p$ residue (a*) of the minor series. Thus, the β-D-Glc$_p$NAc residue (d*) forms a side chain which is linked to the α-L-Rha$_p$ residue (a*) to give a branched tetrasaccharide unit:



Proton NOE relating the anomeric resonances to other protons within the same ring systems provided confirmation of the configurations at the anomeric centers of the glycosyl residues. The occurrence of NOE between H-1 and H-2 of the L-Rha$_p$ and D-Glc$_p$ residues was indicative of all of these

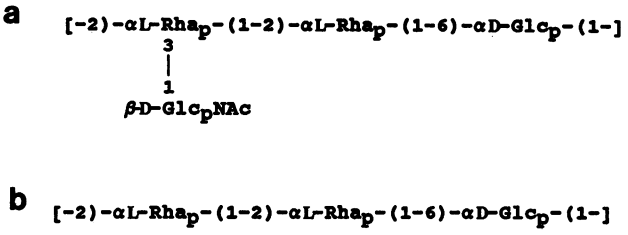


FIG. 4. Structures of the repeating units of the O-PS from serotypes 1, 9, and 11 of *A. pleuropneumoniae*. (a) Tetrasaccharide repeating unit of serotypes 1, 9 (ca. 25%), and 11 O-PS; (b) trisaccharide repeating unit of deaminated serotype 1 and serotype 9 (ca. 75%) O-PS.

residues having α configurations, whereas NOE between H-1 and H-3 and between H-1 and H-5 of the D-Glc$_p$NAc residue confirmed its β configuration (Table 4).

DISCUSSION

The chemical and spectroscopic studies described in the present report reveal a number of structural similarities among the LPS O antigens of *A. pleuropneumoniae* capsular serotypes 1, 9, and 11. Evidence from sugar and linkage analyses, as well as NMR experiments, clearly establishes that the serotypes 1 and 11 of *A. pleuropneumoniae* produce LPS O-PS that have identical branched tetrasaccharide repeating units, and this provides an explanation for the observed serological cross-reactions in ID experiments (Fig. 2a). *A. pleuropneumoniae* serotype 9 LPS O-PS, on the other hand, consists of repeating units having the same trisaccharide linear backbone as the serotypes 1 and 11 O-PS, but the 2,3-di-*O*-substituted Rha$_p$ residues are substituted by the β-D-Glc$_p$NAc groups at the O-3 position, only to the extent of ca. 25%.

These structural conclusions are validated by the results from ID experiments. Antisera to *A. pleuropneumoniae* serotype 9 were precipitated by serotypes 1, 9, and 11 and deaminated 1 O-PS (Fig. 2c). In contrast, serotype 1 antiserum, while showing the homologous and heterologous precipitation reactions, was not precipitated by deaminated serotype 1 O-PS (Fig. 2a [well 1]), indicating an antibody population which is directed solely to epitopes containing the terminal nonreducing β-D-Glc$_p$NAc residues (Fig. 4). Even after absorption with insolubilized serotype 11 LPS, the serotype 9 antiserum is still precipitated by the deaminated serotype 1 O-PS, indicating the presence of two populations of antibodies: those directed toward epitopes containing the terminal β-D-Glc$_p$NAc residues and those directed to linear regions of the backbone polymer (Fig. 4). It is interesting to note that antibody specificities directed toward either the backbone polysaccharide or to an *O*-acetate-substituted variant have been found previously for the CPS of *Escherichia coli* K2 and K62 (22).

At least two alternative structures for the serotype 9 O-PS could account for these observations. These are (i) an O-PS consisting of the trisaccharide backbone randomly substituted by *N*-acetyl-β-D-glucosamine end groups (to the extent of 25%) or (ii) blocks of the trisaccharide repeating units interspersed with blocks of the tetrasaccharide repeating units (in the ratio of 4:1).

A distinction between these two alternatives was made by high-resolution NMR spectroscopy. This required complete assignment of the ^{1}H- and ^{13}C-NMR spectra of the serotype 9 O-PS, and this was achieved by use of homo- and heteronuclear chemical-shift correlation techniques. Subsequently, NOE experiments to establish connectivities between sugars in consecutive oligosaccharide units were employed. This is best illustrated by focusing of the minor series. Thus, irresidue NOEs were observed only between H-1 (4.99 ppm) of α-D-Glc$_p$ residues b* and H-2 (4.27 ppm) of the branched α-L-Rha$_p$ residues a* in consecutive tetrasaccharide units (Fig. 3c). Within the limits of detection of this experiment, no connectivities can be established between H-1 (4.99 ppm) of the α-D-Glc$_p$ residues b* and H-2 (4.11 ppm) of the α-L-Rha$_p$ residue a from a trisaccharide unit. These results are consistent only with the second alternative:

$$[\rightarrow a^*\rightarrow c^*\rightarrow b^*\rightarrow]_m \qquad [\rightarrow a\rightarrow c\rightarrow b\rightarrow]_n$$
$$\uparrow$$
$$d^*$$

Thus, blocks of repeating trisaccharide and tetrasaccharide units are expressed either within a single polymeric chain or as separate polymers in the *n*/*m* ratio of 4:1. The tetrasaccharide unit is polymerized exclusively to give the O-PS of serotypes 1 and 11. Simultaneous expression of two O-PS has been reported for the LPSs of other bacterial species, including *Klebsiella pneumoniae* serotype O1 (33) and *Salmonella boecker* (9).

The current serotyping scheme for *A. pleuropneumoniae* is based on the unique structures of their CPS antigens. Given the results presented here, the serotypes of *A. pleuropneumoniae* could be more rigorously defined by specification of both their CPS and LPS antigenic characteristics. In keeping with the nomenclature introduced for members of the *Enterobacteriaceae* family (20), the term K antigen (from the German word Kapsel) will be used to denote the CPS. Thus, capsular serotypes 1 and 11, both of which express the same LPS O antigen, would be designated K1:O1 and K11:O1, respectively; the O chains of both contain the antigenic factor 1a. The carbohydrate epitope corresponding to this factor would contain the *N*-acetyl-β-D-glucosamine end groups. The CPS serotype 9 would be designated K9:O9, its O chain containing the antigenic factors 1a and 9a, in which the repeating trisaccharide backbone gives rise to the antigenic factor 9a. Furthermore, *A. pleuropneumoniae* capsular serotypes 3 and 8 also have identical O chains (27); these serotypes would be designated K3:O3 and K8:O3, respectively.

It is anticipated that the proposed serotyping scheme involving a description of both the K and O antigens will provide an adequate description of all *A. pleuropneumoniae* strains.

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